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RESEARCH ARTICLE

Construction and Evaluation of the Toxoplasma gondii DNA vaccine targeting DEC-205

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A B S T R A C T

Toxoplasmosis, caused by *Toxoplasma gondii* (*T. gondii*), remains a significant public health problem. Recently, it has been shown that the antigens targeting the dendritic cells (DCs) is a promising way to enhance the immune responses against pathogens and tumors. In this study, we constructed and evaluated a new DNA vaccine of *T. gondii*, based on the major surface antigen SAG1 of *T. gondii* and an antibody single-chain fragment variable (scFv) directed against the mouse DEC 205, an endocytic receptor expressed on the surface of DCs and other cells. The constructs were tested *in vitro* to express the encoded proteins, then the cellular and humoral immune responses were analyzed, and the protective efficacy of the vaccine was evaluated. Our results showed that the plasmid encoding the fusion of DEC-205 scFv with SAG1 significantly enhanced the SAG1-specific immune response and DC targeting could elicit the Th1 type immune response, evidenced by the high level of IFN-γ, IL-2 and IgG2a. Therefore, we showed evidence in this research that DEC-205 can be exploited in developing an effective *T. gondii* vaccine.

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INTRODUCTION

Parasitic diseases are a major trouble in many parts of the world (Lin *et al*., 2020; Chen *et al*., 2020; Mohsin *et al*., 2021 a,b). Among parasites, *T. gondii* is an obligate intracellular protozoan parasite of global importance, which causes toxoplasmosis in animals and humans (Zhao *et al*., 2012). This parasite is thought to have affected one-third of the world's human population. *T. gondii* can be transmitted through horizontal and vertical transmission pathways in the form of tachyzoites, bradyzoites, and sporozoites. Many infected people with a healthy immune system are asymptomatic and have only mild flu-like myalgia symptoms (Wang *et al*., 2016). However, when a primary or reactivated infection occurs during pregnancy, serious health consequences such as abortion or severe congenital disabilities in the fetus, including mental retardation, blindness, and hydrocephalus, may occur (Pfaff *et al*., 2014). Reactivation or primary infection in patients with HIV infection, organ transplant recipients and cancer patients with immunocompromised conditions may lead to severe complications, such as encephalitis or pneumonia (Robert-Gangneux and Dardé, 2012). In addition to human diseases, *T. gondii* can cause abortion, neonatal death and loss of offspring in livestock, especially in pigs, sheep and goats, posing a severe threat to the sustainable agricultural economy (Hill and Dubey, 2013).

The surface antigens of *T. gondii* tachyzoites, including SAG1, SAG2, and SAG3 are considered candidates for putative vaccines (Wang and Yin, 2014). SAG1 and SAG2 proteins are mainly engaged in host cell invasion and are responsible for triggering immune responses to *T. gondii.* SAG1 is an immunodominant antigen in the tachyzoite stage which is retained among various *T.gondii* strains. SAG1 comprises epitopes of T and B cells (Wang *et al*., 2013), and subunit vaccines based on SAG1 have been shown to produce an efficient

and long-lasting immune response in immunized mice (Chuang *et al*., 2013).

DCs are antigen-presenting cells (APCs) that perform a crucial part in initiating and regulating adaptive immunity due to their unique capability to capture, process and show different antigens to CD_8^+ and CD_4^+ T cells (Sehgal *et al*., 2014). Targeting antigens to DCs by DC receptor-specific mAbs is an approach that can be used to boost cellular and humoral immunity to the particular antigens. DEC-205 has been extensively employed for DC-based vaccines in murine, human and poultry studies among these receptors. DEC-205 belongs to the family of mannose receptors (MR), a C-type lectin superfamily, expressed in macrophages, B cells, and epithelial thymus, lung and intestinal cells, especially DCs (Bozzacco *et al*., 2007). Like other members of the mannose receptor family, DEC-205 is a membrane receptor that can enter the cell by clathrin-mediated endocytosis. DEC-205 can precisely capture antigens as a pattern recognition receptor on the surface of DCs, process and present them to the immune system, and induce an immune response. The targeted DEC-205 strategy has proved successful against viral (Volckmar *et al*., 2017), bacterial (Do *et al*., 2012), and parasitic infections (Lakhrif *et al*., 2018).

Several studies have used the whole monoclonal antibodies to target DC antigens. Meanwhile, scFv of the antibodies were less frequently used. However, the use of scFv for antigen targeting has many advantages. The small size and lack of Fc fragment of scFv improves tissue penetration and reduces the immune response against the antibody itself (Demangel *et al.*, 2005).

The present research is aimed to develop a novel vaccine strategy against *T. gondii* infection based on SAG1 and scFv of DEC-205 antibody. We examined the cellular and humoral immune responses activated by the targeted and the untargeted DNA vaccines. We found that DEC-205 antibody targeting SAG1 to DCs through scFv represents an encouraging protocol to improve defence against *T. gondii* infection.

MATERIALS AND METHODS

Mice and *T. gondii* strain: Female BALB / c mice aged 3-5 weeks old were purchased from Wu's Experimental Animal Center, Fuzhou, China. The animals were used according to Fujian Agriculture and Forestry University approval by the Ethics Committee (No. 2018-6-20). *T. gondii* RH strain tachyzoites were retained by the serial intraperitoneal passage in the mice.

Construction of recombinant DNA vaccines: SAG1, ScFv of anti-DEC205, derived from NLDC-145 mAB (scFvDEC205), and scFvDEC205-SAG1 were codon-optimized and synthesized by Sangon Biotech Company (Shanghai, China). After digestion with EcoRI and XhoI, they were ligated into pVAX1 to construct recombinant plasmids pVAX1-scFvDEC205, pVAX1-SAG1, and pVAX1-scFvDEC205-SAG1. Then the plasmids were confirmed by restriction enzyme digestion and quantified by spectrophotometry.

Expression of the plasmids *in vitro*: The plasmids were transfected into 293T cells using Trans Intro TM EL Transfection Reagent (Trans Gen Biotech, Beijing, China), as specified by the manufacturer's instructions when the cells were cultured in six-well plates for 12 h. The expression of SAG1 proteins in the 293T cells was confirmed using indirect immunofluorescence and western blot assays at 24 h post-transfection as described previously (Huang *et al*., 2018).

Immunization and challenge: Five separate groups were taken, each group having 20 mice. The pVAX1-scFvDEC205, pVAX1-SAG1, pVAX1-scFvDEC205-SAG1, pVAX1, and PBS were named to the groups. Each mouse thigh muscle was injected with 50 µg of the corresponding nucleic acid vaccine, and a total of three immunizations were performed two weeks apart. The control group was treated with PBS.

At 14 days after each immunization, the blood was extracted from the eyelids, and the serum was isolated and placed in a refrigerator at -20°C, 1×10^4 toxoplasma RH strain tachyzoites were intraperitoneally injected into the mice on the 14 days after the third immunization, the mice's survival period was observed and noted, regularly.

Measurement of humoral antibodies response: The ELISA was conducted in the serum to examine specific SAG1 antibodies. SAG1 protein was diluted to 4 µg / ml with 50 mM carbonate buffer (pH 9.6), applied to a 96-well ELISA plate at 100 µl per well, and coated at 4 ° C overnight. The plates were washed with PBS-T (0.05% v / v Tween 20) and blocked in PBS-T with 5% skimmed milk. In PBS-T, serum dilution (1:100) was done in 2% skimmed milk, and 100 µl diluted serum was added to the plates and incubated at 37 ° C for 1 h. Then the plates were washed again and incubated at 37 ° C for 1 h with 100 µl HRP-labeled goat anti-mouse IgG, IgG1, and IgG2a (both at 1:5,000, Bethyl Laboratories, Inc, Montgomery, American), after washing, the plates were incubated with tetramethylbenzidine (TMB) for 10 min at room temperature in the dark and the reaction stopped by 2 M H_2SO_4. A microplate reader (Tecan, Switzerland), calculated the OD450 value of each sample.

Cytokine assays: At 14 days after the third immunization, Sera was collected from the mice. Interferon-gamma (IFN-γ), interleukin-4 (IL-4), interleukin-10 (IL-10), and interleukin-12 (IL-12) were tested using ELISA kits as instructed by the manufacturer (4 A Biotech, Beijing, China). A microplate reader (Tecan, Switzerland) was used to measure the OD at a wavelength of 450 nm. The sample serum concentrations of cytokines IL-4, IL-10, IL-12, and IFN-γ were calculated based on the standards and OD values.

Statistical analysis: Using Graph Pad Prism Program 7.0, the statistical significance was analyzed. Statistical analysis was performed by one-way ANOVA followed by multiple comparison tests by a Tukey and Student's t-test. The $P<0.05$ has been assumed as statistically significant.

RESULTS

Construction and verification of DNA vaccines: The recombinant plasmids were obtained and verified by restriction enzyme digestion and sequencing. PVAX1-SAG1, pVAX1-scFvDEC205, and pVAX1-scFvDEC205-SAG1 were digested with EcoRI and XhoI. The digested products were visualized by 1% agarose gel electrophoresis, and the migration of the DNA fragments was following the expected sizes, as shown in Fig. 1A. The plasmid sequencing results provided by Sangon Biotech Company (Shanghai, China) were identical to the known gene sequences, and the fused genes were in the same open reading frame. Thus, it was shown that the recombinant plasmids were successfully constructed.

The plasmids were transfected into 293 T cells, and the protein expression was determined by Western blotting and IFA. The results of Western blotting revealed bands at about 35 kDa, 31 kDa, and 66 kDa for pVAX1-SAG1, pVAX1-scFvDEC205, and pVAX1-scFvDEC205-SAG1, as shown in Fig. 1B, confirming that the recombinant plasmids expressed the encoded proteins containing the 6x His tag. IFA results are shown in Fig. 1C, using FITC to visualize the expression of SAG1 and SAG1 fusion proteins in 293T cells, In the cells transfected with the vaccine plasmid pVAX1-SAG1, pVAX1-scFvDEC205, and pVAX1-scFvDEC205-SAG1, green fluorescence was observed. Moreover, in the cells transfected with the control pVAX1 vector (Fig. 1C), no green fluorescence was observed.

Humoral immune responses to the DNA vaccines: ELISA assessed the serum antibody levels IgG, IgG1, and IgG2a. As illustrated in Fig. 2A, the serum of mice immunized with plasmids pVAX1-SAG1 and pVAX1-scFvDEC205-SAG1 ($P<0.01$) against other classes (PBS, pVAX1, and pVAX1-scFvDEC205) observed significantly higher levels of IgG antibodies, and with successive immunizations, the rates of antibodies increased. As probable, the control mice did not detect any increase in the antibody levels (Fig. 2A); among them, the scFvDEC205-SAG1 group had the highest level of IgG antibody. Besides, IgG1 and IgG2a were found in the sera of plasmid-vaccinated mice pVAX1-SAG1 and pVAX1-scFvDEC205-SAG1, showing a mixed anti-SAG1 IgG1 / IgG2a profile (Fig. 2B, C).

Cytokine production: Sera samples after the third immunization were used to detect the level of cytokines IFN-γ, IL-4, IL-10, and the IL-12 produced in the groups (Table 1). IFN-γ and IL-12 levels in pVAX1-SAG1, pVAX1-scFvDEC205-SAG1 groups were significantly higher than those in the PBS, pVAX1 and pVAX1-scFvDEC205 groups. There were significant differences between the pVAX1-SAG1 and pVAX1-scFvDEC205-SAG1 groups ($P<0.05$). For IL4 and IL10 levels, significant differences we're observed among pVAX1-scFvDEC205, pVAX1-SAG1 and pVAX1-scFvDEC205-SAG1 groups. The results showed that the pVAX1-SAG1 and pVAX1-scFvDEC205-SAG1 DNA vaccines could enhance the mice's secretion of type 1 cytokines.

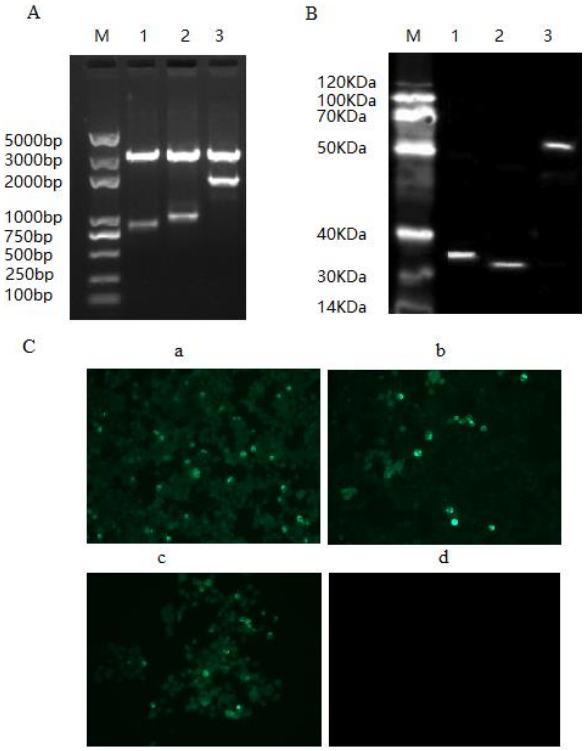

Fig. 1: Identification of the recombinant plasmids and expression of the proteins by restriction endonuclease digestion, western blot and IFA. (A) Recombinant plasmids, pVAX1-SAG1 (lane 1), pVAX1-scFvDEC205 (lane 2) and pVAX1-scFvDEC205-SAG1 (line 3) were digested by EcoRI and XhoI. (B) Proteins expression, pVAX1-scFvDEC205 (lane 1), pVAX1-SAG1 (lane 2) and pVAX1-scFvDEC205-SAG1 (line 3) were established by the Western blotting method with anti-His 6 monoclonal antibodies. (C) 293T cells were transfected with the plasmids, pVAX1-scFvDEC205 (a), pVAX1-SAG1 (b) and pVAX1-scFvDEC205-SAG1 (c) and pVAX1 (d), and IFA confirmed the expression of the proteins with anti-His 6 monoclonal antibodies as the primary antibody. The DNA marker is from Trans Gen Biotech (China), and the protein ladder is from Fermentas (USA).

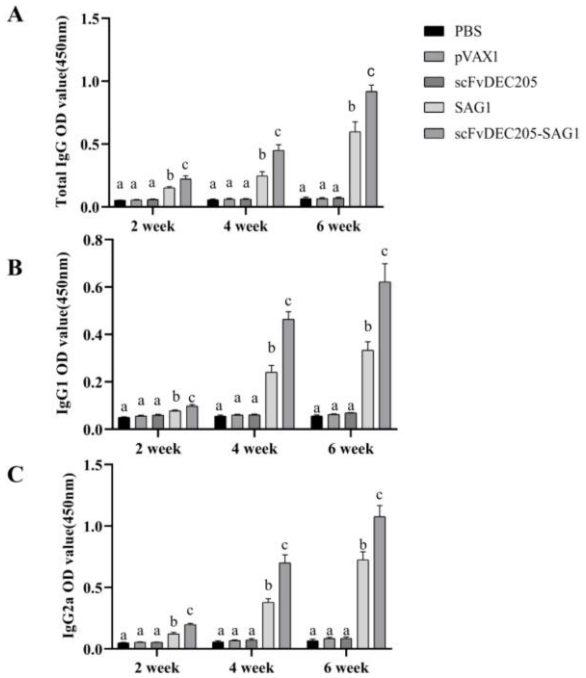

Fig. 2: Antibody immune responses induced by DNA vaccines. Total IgG level (A) and IgG subclass IgG1 (B) and IgG2a (C) in the sera of BALB/c mice immunized with PBS, pVAX1, pVAX1-scFvDEC205, pVAX1-SAG1 and pVAX1-scFvDEC205-SAG1 on weeks 2, 4, 6. Means with different letters were significantly different between treatment groups ($P<0.05$).

Table 1: The concentration of cytokines IL-4, IL-10, IL-12, and IFN-γ in serum after the third immunization (pg/ml).

Group	IFN-γ	IL-4	IL-10	IL-12
PBS	80.42±4.76[a]	66.65±3.76[a]	54.31±1.38[a]	62.87±5.86[a]
pVAX1	82.66±3.71[a]	67.16±3.21[a]	56.28±2.09[a]	63.38±4.11[a]
pVAX1-scFvDEC205	84.47±4.67[a]	67.36±3.95[a]	55.72±3.36[a]	62.91±4.50[a]
pVAX1-SAG1	145.95±3.90[b]	97.87±7.23[b]	93.31±4.91[b]	95.92±2.88[b]
pVAX1-scFvDEC205-SAG1	187.16±3.16[c]	123.13±3.28[c]	119.79±5.06[c]	113.94±4.03[c]

The data are represented by average ±standard deviation, and there are significant differences in the representation of different letters in the same list (P<0.05).

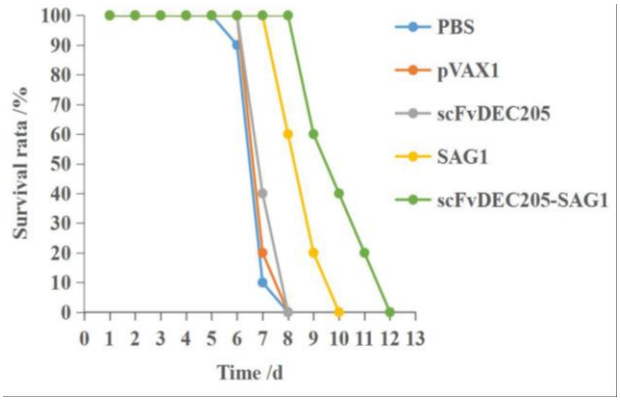

Fig. 3: Survival curve of mice after challenge infection with *T. gondii* RH strain. Mice were challenged with 10^4 tachyzoites of the RH strain intraperitoneally two weeks after the third immunization.

Protection against lethal toxoplasmosis in mice: For the five groups, the survival curves are presented in Fig. 3. The control group mice (PBS, pVAX1 and pVAX1-scFvDEC205) died within 8 days of the experiment. The life period of mice in the pVAX1-scFvDEC205-SAG1 group was more extended than in other groups, indicating that the mice receiving the *T. gondii* SAG1 subunit vaccine targeting DEC-205 were more protected than the controls. Notably, the mice treated with the SAG1 subunit vaccine targeting DEC-205 lived four days more than the blank control group. This indicated the pVAX1-scFvDEC205-SAG1 vaccine could target the SAG1 antigen to antigen-presenting cells, thereby mediating the efficient internalization of antigen by antigen-presenting cells, then induce a strong specific immune response.

DISCUSSION

Currently, there is no ideal therapeutic drugs and vaccines for treating toxoplasmosis, and drugs are just sufficient for the tachyzoites. So, there is a great need to develop a valuable vaccine against *T.gondii* with effective mode. In this research, a DNA vaccine encoding TgSAG1 and DEC205 antibody single-chain fragment variable was constructed. Its ability to evoke remarkable immune responses and protect BALB/c mice against *T. gondii* RH strain infection was studied.

The DEC-205 is a C-type lectin receptor that expresses itself on immature DCs and various epithelial cells (Sehgal *et al*., 2014). It has been shown that targeting antigens to DEC-205 by specific antibody-antigen complexes outcomes in active uptake and presentation of antigens on complexes MHC I and MHC II, which results in antigen-specific CD_4^+T and CD_8^+ cells being activated, respectively (Do *et al*., 2008). An effective strategy for developing a protein vaccine based on the antibody TgSAG1 and DEC-205 has been documented, and the efficacy of this vaccine against chronic toxoplasmosis infection has been studied.

The previous research showed that DC targeting enhanced both local and systemic cellular immune and humoral responses and enhanced Th1 response profile by developing more efficiently IgG2a, IL-2, IFN-γ, and IgA (Lakhrif *et al*., 2018). Our research extended this strategy to the DNA vaccine against acute *T. gondii* infection. The results showed that the pVAX1-scFvDEC205-SAG1 vaccine could increase the survival time of *T. gondii* tachyzoites (RH strain) challenged BALB / c mice, as compared to other groups.

Antibodies play an important role against pathogens by stimulating the macrophages and eventually binding the *T.gondii* surface protein to occupy the invasion of *T.gondii* into the host cells (Pifer and Yarovinsky, 2011). Here, the higher levels of IgG antibody in the pVAX1-scFvDEC205-SAG1 group were encouraged compared to the levels in other groups (P<0.05).

Further analysis of the IgG1 and IgG2a responses has established a mixed humoral immune response. The level of IgG2a is higher than the level of IgG1, which indicates that our DC-targeted vaccine may elicit a Th1-biased response. This result matches with those described in previous study (Wang *et al*., 2015). Cellular immunity plays a significant role in anti-toxoplasma immunity, especially in the early stage of acute infection. Toxoplasma-mediated cellular immunity includes activating antigen-specific T cells and innate immune cells (Dupont *et al*., 2014). In mice, Toxoplasma infection quickly induces myeloid differentiation primary response 88 (MyD88)-dependent production of IL-12 in DC with myeloid. In the early stages of toxoplasmosis, it is essential for parasite control to have IFN- γ provided by NK cells and type I congenital lymphocytes (Wang *et al*., 2019).

DC and macrophage production against antigens are required to activate and amplify parasite-specific $CD4^+$ and $CD8^+$ T cells. Such type of immune cells is also responsible for the production of IFN-γ. Finally, they play a significant role in cerebral toxoplasmosis regulation. We observed that the DC-targeted vaccine could produce higher IFN- γ, and IL-12, belonging to the Th1 family of cytokines responsible for resistance against infection by T.gondii correspondingly with a previous study (Sturge and Yarovinsky, 2014). IL-10 and IL-4 are associated with Th2 response; IL-4 can promote B cell generation, differentiation, and maturation, thus induce the antibody response (Bao and Cao, 2014). IL-10 is an important anti-inflammation factor; it can reduce the inflammatory response caused by the parasites.

Conclusions: Our study showed that the DNA vaccine at DC can induce strong humoral and cellular immune

responses and can provide partial protection to the hosts after the lethal challenge of *T. gondii*. This research demonstrates the promise of a DC-targeted DNA vaccine as a novel approach to toxoplasmosis infection and could be implemented in developing *T. gondii* vaccines.

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Authors contribution: The work was established and supervised by GWY. The data were experimented and analysed by RC, JJP, MM, XHH, XLL, ZJH. GWY and MM wrote the paper, LAM polished this paper. All authors read and approved the final manuscript.

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